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Reevaluation of 3′E_κ Function in Stage- and Lineage-Specific Rearrangement and Somatic Hypermutation

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Summary

Transgenic studies have led to the conclusion that the 3′E_κ enhancer functions to suppress κ variable region gene assembly in T lineage cells and in progenitor B cells and have also implicated 3′E_κ as a critical element in promoting somatic hypermutation of κ variable region genes. To assess the role of the endogenous 3′E_κ, we assayed these processes in mice homozygous for mutations in which the 3′E_κ sequences were deleted by the *loxP*/Cre method (3′E_κΔ/Δ mice). In contrast to transgenic findings, we found that deletion of the endogenous 3′E_κ did not deregulate κ gene rearrangement in T lineage cells or in pro-B cells. Furthermore, immunization of the 3′E_κΔ/Δ mice led to the generation of specific antibodies with mutation patterns typical of affinity maturation, showing that there is no absolute requirement for the 3′E_κ with respect to somatic mutation of endogenous κ genes.

Introduction

Immunoglobulin heavy and light chain variable region genes and T cell receptor variable region genes are assembled, respectively, in differentiating B and T lineage cells from component V, D, and J segments (reviewed by Okada and Alt, 1994). Immunoglobulin κ light chain (LC) variable region genes are assembled subsequent to Ig heavy chain (HC) variable region genes during B cell differentiation and are not assembled in T lineage cells (reviewed by Lansford et al., 1996; Gorman and Alt, 1998). As the V(D)J recombination process is effected by a common enzymatic machinery, this lineage-specific rearrangement is mediated, at least in part, by *cis*-acting regulatory elements, such as transcriptional enhancers, that modulate accessibility of the substrate variable region gene segments (reviewed by Sleckman et al., 1996). The Igκ locus includes two known B cell–specific transcriptional enhancer elements: the intronic κ enhancer/matrix attachment region (iE_κ/MAR) located in the J_κ-C_κ intron and the 3′ κ-enhancer (3′E_κ) located 8 kb downstream of the C_κ gene (Emorine et al., 1983; Picard and Schaffner, 1984; Queen and Stafford, 1984; Meyer and Neuberger, 1989).

Transfection and transgenic studies have shown that both the iE_κ/MAR and the 3′E_κ promote transcription of rearranged κ genes (Staudt and Lenardo, 1991). However, gene-targeted mutational analyses have shown that deletion of the iE_κ/MAR results in a major impairment in κ gene rearrangement with no effect on rearranged κ gene expression (Xu et al., 1996). Transgenic reporter gene studies have shown that 3′E_κ becomes active during the late pro-B to pre-B cell stages and becomes even more active upon B cell activation (Meyer et al., 1996). The 3′E_κ has been implicated as necessary for optimal expression of rearranged κ genes, as addition of the 3′E_κ to rearranged κ transgenes that included the iE_κ/MAR resulted in enhanced expression levels, which were sufficient to block endogenous κ gene rearrangement (Meyer et al., 1990; Blasquez et al., 1992). Gene-targeted mutation studies further showed that deletion of the 3′E_κ resulted in a substantial impairment of κ gene expression in resting B cells, with a less dramatic effect on κ gene rearrangement (Gorman et al., 1996). Together, these types of studies suggested that the 3′E_κ and the iE_κ/MAR have overlapping activities, with each having a more central role in certain processes.

Recent transgenic studies have led to the surprising conclusion that the 3′E_κ sequence may play a negative role in regulating κ gene rearrangement (Hiramatsu et al., 1995; Hayashi et al., 1997). Thus, mice that carry transgenic κ recombination substrates with only the intronic κ enhancer and no 3′κ enhancer (or a 3′E_κ that lacked the binding site for the transcription factor PU.1) were found to rearrange the transgenic V_κ genes in both B and T lineage cells, leading to the conclusion that the 3′E_κ serves as a negative regulator of κ gene rearrangement in developing T cells (Hiramatsu et al., 1995). Rearrangement of the majority of endogenous V_κ genes in normal mice occurs at the pre-B cell stage of development, subsequent to Ig HC gene rearrangement (reviewed by Lansford et al., 1996). However, transgenic κ LC gene minilocus recombination substrates that lacked 3′E_κ were found to undergo rearrangement at the early pro-B to late pro-B cell stage, simultaneous with HC gene rearrangement (Hiramatsu et al., 1995). Furthermore, nucleotide sequence analysis of the V_κ-J_κ joins in these substrates revealed a high frequency of N insertions, confirming that these rearrangements must have occurred at the pro-B cell stage, in which terminal deoxynucleotidyl transferase (TdT) activity is high. In addition, the pro-B/pre-B rearrangement specificity was also lost when the 8.4 kb region between C_κ and 3′E_κ was deleted, suggesting the existence of an additional suppressive element that regulates pro-B/pre-B rearrangement specificity (Hiramatsu et al., 1995; Hayashi et al., 1997).

After assembly and expression of functional Ig HC and LC genes, B lineage cells mature in the bone marrow and then migrate into the periphery where, upon interaction with cognate antigens, they can undergo further maturation (reviewed by Milstein and Neuberger, 1996). Such activation of the B cells results in the formation of germinal centers and the activation of a somatic hypermutation process that introduces mutations into the HC and LC variable region genes, allowing further affinity

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maturation of the antibody response (Liu et al., 1989; Berek et al., 1991; Jacob and Kelsoe, 1992; MacLennan, 1994). Studies employing rearranged κ LC transgenes have suggested that both the 3′E_κ and the iE_κ/MAR are critical for allowing the somatic hypermutation process to act on linked V_κ genes (Sharpe et al., 1991; Betz et al., 1994). Deletion of either enhancer from the constructs resulted in a severe drop of mutation frequency. However, the extent of mutation in constructs lacking the 3′E_κ appeared linked to the level of κ transgene expression in mature and activated B cells (Meyer et al., 1996; Goyenechea et al., 1997). Because such expression varied with respect to different integration sites of the transgene, it was difficult to directly assess the function of the 3′E_κ in promoting κ expression versus specifically recruiting the somatic mutation machinery.

To date, all information regarding potential functions of the 3′E_κ and the iE_κ with respect to lineage and stage specificity and somatic mutation has come from assays of transfected cell lines and mice with transgenes containing rearranged or unrearranged κ miniloci under the control of one, the other, or both enhancers. However, the role of these enhancers in regulating such functions has not been studied in the context of the endogenous locus. Recently, we generated two mutations of the mouse germline in which the 3′E_κ was replaced by either a *neo*^{r} gene (3′E_κN) or by a *loxP* sequence (3′E_κΔ) (Gorman et al., 1996). Mice homozygous for the 3′E_κΔ mutation (3′E_κΔ/Δ) had decreased numbers of splenic B cells and reduced levels of surface κ expression. In addition, κ to λ ratios were severely reduced to 2:1 as compared to 20:1 in wild-type (wt) littermates. The homozygous 3′E_κN mutation (3′EκN/N) resulted in a similar but more pronounced phenotype (with κ to λ ratios of 1:1). One possible interpretation of this effect is that the expressed *neo*^{r} cassette somehow neutralizes the activities of a complementary *cis*-acting element such as the iE_κ/MAR.

Here, we have utilized the 3′E_κΔ/Δ mutant mice to directly assess putative 3′E_κ functions with respect to stage and lineage specificity and with respect to promoting somatic hypermutation following specific immunization.

Results

B/T Specificity of κ Gene Assembly Is Retained in 3′E_κ Mutant Mice

To test whether the endogenous 3′E_κ is required for suppression of κ gene rearrangement in T lineage cells, we assayed genomic DNA (gDNA) from purified thymocytes and peripheral T cells of wt and 3′E_κΔ/Δ mice for the presence of V_κ-J_κ rearrangements. For this purpose, we utilized a polymerase chain reaction (PCR) assay that employed a degenerate V_κ primer and a J_κ2-specific primer (Figure 1A) (Schlissel and Baltimore, 1989). We first tested gDNA samples isolated from splenic T cells and total spleen cells with amounts ranging from 0.1 to 1000 ng per reaction. While total spleen cell DNA from wt and 3′E_κΔ/Δ mice generated readily visible κ rearrangement PCR products with gDNA amounts as low as 1 ng (Figure 1B lanes 11–20), no κ rearrangement products were detected with even the highest amount of purified splenic T cell gDNA from either wt or 3′E_κΔ/Δ

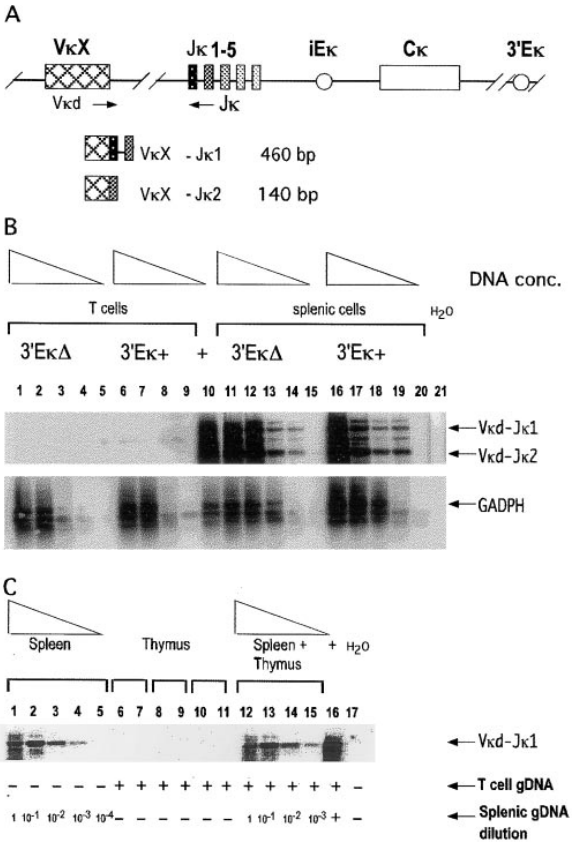

Figure 1. Analysis of κ Gene Rearrangement in Thymic and Splenic T Cells of 3′E_κΔ/Δ and Wt Littermates

(A) Schematic map showing the structure of the κ locus and description of κ-specific PCR primer locations and expected products.
(B and C) PCR assay for κ gene rearrangements in Δ/Δ and control (+/+) DNA samples. V_κ-J_κ junctions were amplified in 30 cycles with a degenerate V_κ primer and a specific J_κ2 primer (as shown in [A]) from genomic DNA (gDNA) samples generated from total spleen cells and FACS-sorted CD3$^+$ splenic and thymic T cells of Δ/Δ4 (B) or Δ/Δ23 and Δ/Δ24 (C) mice and of +/+5 (B) and +/+17 (C) wt mice. In both panels, GADPH genes were amplified in 22 cycles with specific primers using the same gDNA samples. PCR V_κ-J_κ1 and V_κ-J_κ2 rearrangement products were detected with a J_κ probe as described in Experimental Procedures and GADPH products were detected with a GADPH-specific cDNA probe. In (B), lanes 1–5 and 6–9 contain PCR products obtained with 10-fold serial dilutions of splenic T cell gDNA derived from, respectively, mouse Δ/Δ4 and +/+5, starting at 1 μg. Lanes 10 and 21 are control lanes obtained with, respectively, 2 μg of +/+5 splenic gDNA and water. Lanes 11–15 and 16–20 contain PCR products obtained with 10-fold serial dilutions of total splenic gDNA derived from, respectively, Δ/Δ4 and +/+5 mice, starting at 1 μg. In (C), lanes 1–5 contain PCR products obtained with 10-fold serial dilutions of splenic gDNA derived from the Δ/Δ23 mouse (starting at 500 ng), lanes 6–7 contain PCR products obtained with 500 ng and 100 ng of thymic Δ/Δ23 mouse gDNA, lanes 8–9 contain PCR products obtained with 500 ng and 100 ng of thymic +/+17 gDNA, lanes 10–11 contain PCR products obtained with 500 ng and 100 ng of thymic Δ/Δ24 mouse gDNA, and lanes 12–15 contain splenic Δ/Δ23 mouse gDNA in the same dilution range as in lane 1–5 with the addition of thymic Δ/Δ23 mouse gDNA (500 ng). Lanes 16 and 17 are, respectively, splenic Δ/Δ26 gDNA and a water control.

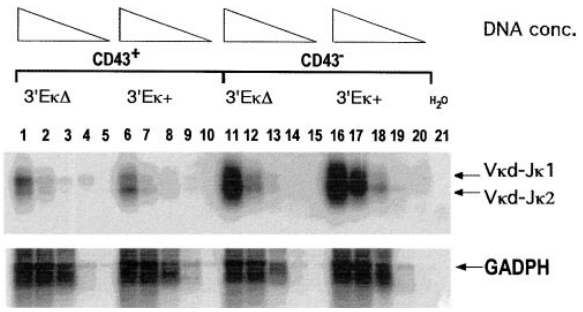

Figure 2. Analysis of κ Gene Rearrangement in Pro- and Pre-B Cells of 3′E_κΔ/Δ and Wt Littermates

V_κ-J_κ junctions were amplified in 30 cycles with a degenerate V_κ primer and a specific J_κ2 primer using genomic DNA (gDNA) samples derived from FACS-sorted pro-B and pre-B cells from bone marrow of mouse Δ/Δ8 and mouse +/+7. GADPH genes were amplified in 22 cycles with specific primers using the same gDNA samples. Lanes 1–5 and 6–10 contain PCR products obtained with 10-fold serial dilutions of pro-B cell gDNA derived from, respectively, mouse Δ/Δ8 and mouse +/+7. Lanes 11–15 and 16–20 contain PCR products obtained with 10-fold serial dilutions of pre-B cell gDNA derived from, respectively, mouse Δ/Δ8 and mouse +/+7, and lane 21 is a control PCR that contains no gDNA.

mice (Figure 1B, lanes 1–9). Next, we amplified κ rearrangements from purified thymic ($CD3^+$) T cell gDNA with the same set of primers; again, no V_κ-J_κ1/2 joins were detected at any concentration of either wt or mutant thymocyte gDNA (Figure 1C, lanes 6–11). To further test the detection sensitivity for κ rearrangements, varying amounts of spleen cell gDNA were mixed with 500 ng of purified thymocyte gDNA and then subjected to the amplification procedure. Similar levels of κ rearrangements were detectable with and without the addition of thymocyte gDNA, indicating that the thymocyte gDNA was in no way inhibitory to the PCR process (Figure 1C, compare lanes 1–5 to lanes 12–15). We conclude that mice homozygous for a germline deletion of 3′E_κ show no evidence for deregulation of V_κ to J_κ recombination in T lineage cells.

Stage Specificity of κ Gene Recombination Is Maintained in 3′E_κ Mutant Mice

To evaluate the putative suppressive role of the 3′E_κ region on the rearrangement of endogenous κ genes at the pro-B/pre-B stage of development, we assayed for rearrangements of the κ locus in pro-B cells of 3′E_κΔ/Δ mice. For this purpose, pro-B and pre-B cells were purified by FACS sorting of, respectively, $B220^+$, $CD43^+$, IgM^- and $B220^+$, $CD43^-$, IgM^- bone marrow cells, from 3′E_κΔ/Δ and wt littermates. V_κ-J_κ rearrangements were then amplified from gDNA derived from both purified populations by PCR with the degenerate V_κ primer in combination with a J_κ2 (or J_κ5) primer. As expected, we observed high levels of V_κ-J_κ rearrangements in DNA samples from the pre-B cell fractions of wt and mutant mice (Figure 2, lanes 11–20). However, we found only low levels of V_κ-J_κ rearrangements in the pro-B cell DNA samples derived from either wt or mutant mice, and the level of the V_κ-J_κ rearrangements in these samples was similar (Figure 2, lanes 1–10).

Nucleotide sequence analysis of pro-B and pre-B cell–derived κ rearrangements from mutant B lineage cells demonstrated the absence of any apparent N region insertions (Figure 3). Because it is sometimes difficult to find the correct V_κ germline counterpart sequence for isolated V_κ-J_κ rearrangements, we also determined the nucleotide sequence of a set of V_κOx family specific κ rearrangements, where the contributing V_κ gene segment could be identified with certainty. For this purpose, V_κOx rearrangements were amplified from pro-B and pre-B cells using a V_κOx family specific primer together with a J_κ2 or J_κ5 primer. All V_κOx-J_κ junctional sequences obtained from the mutant B cells were aligned with the germline V_κOx1 sequence; again we observed no increased N region additions in the V_κOx rearrangements isolated from the 3′E_κΔ/Δ pro-B or pre-B cell populations (Figure 3). Furthermore, we confirmed this result via analyses of V_κOx-J_κ5 transcripts isolated from PNA^{high} splenic B cells after immunization with oxazolone (see below). Together, our findings indicate that deletion of 3′E_κ does not promote premature V_κ gene recombination within the endogenous κ locus at the pro-B cell stage.

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IGKBM45                                VkD-Jk junction      JK
K02417 tttattactgtatgcaaggtctagaatatcc
32     ................ca.............          gtacacgttcggaggg jk2
S74547 tttattatctgtgctcaaatctagaacttcc
34     ............................              gtacacgttcggaggg jk2
M34885 ttattactgtatgcaacatctagaatatccttt
36     ..c......a..............ct.....           tacacgttcggaggg jk2
U39901 tttattactgctttcaaggttcacatgttcc
65     ............................              gtaaacgttcggagga jk2
L78682 tttatttctgctctcaaagtacacatgttcc
35     ........a.....................            gtggacgttcggtgga jk1
IGKBM28
X86549 cttattactgccaccagtatcatcgttccccaccc
21     ...................................            acgttcggtggn jk1
X86548 cttattactgccatcagcggagtagttaccca
22     ...................................          tcacgttcggtgct jk5
Af04330tttattactgccagcagtggaattatcctctta
23     ...........................              gctcacgttcggtgct jk5
IGKBM45                          Vkox-Jk junction
                                                        JK
VkOx1: CTTATTACTGCCAGCAGTGGAGTAGTAACCCACCCA N
7      .........t..a...........t....          gctcacgttcggtgct jk5
17     ............t...c........t....             cacgttcggtgct jk5
14     ................aa........t.....                cggtgct jk5
7b     ......................a....t....          gctcacgttcggtgct jk5
16     ...................................          tcacgttcggtgct jk5
13     ......t......t............t.....       attcacgttcggctcg jk4
17b    ............t...c........t....          attcacgttcggctcg jk4
16b    ......................g..t.....            ttcacgttcggctcg jk4
72     ................aa........t....             tacacgttcggaggg jk2
74     .........t..a............t....          gtacacgttcggaggg jk2
75     .....ct.....t.............t.....            acacgttcggaggg jk2
66     ......................g..t.....          gtacacgttcggaggg jk2
71     ...................................      gtacacgttcggaggg jk2
38     ...................................      gtacacgttcggaggg jk2
48     ..............................tc.....        ggacgttcggtgga jk1
67     ..................atca....t.........        ggacgttcggtgga jk1
IGKBM26
VkOx1: CTTATTACTGCCAGCAGTGGAGTAGTAACCCACCCA
                                                        JK
2b     ......................g.......          gctcacgttcggtgct jk5
6b     ................c.........t..          gctcacgttcggtgct jk5
11b    ..................g...a.tt....           ctcacgttcggtgct jk5
9b     ............................t.....            cacgttcggtgct jk5
1b     ..........t.....aa........t.........            gttcggtgct jk5
5b     ...................................      gctcacgttcggtgct jk5
4b     ......................g..t.....            tcacgttcggtgct jk5
9b     ...................................      gctcacgttcggtgct jk5
12b    ......................g.......          gctcacgttcggtgct jk5
14b    ..................................  t       acgttcggtgct jk5
```

Figure 3. Nucleotide Sequences of V_κ-J_κ Junctions Amplified from $CD43^+/IgM^-$ and $CD43^-/IgM^-$ Bone Marrow B Cells of 3′E_κΔ/Δ Mice

$CD43^+/IgM^-$ and $CD43^-/IgM^-$ bone marrow cells were isolated from three mutant 3′E_κΔ/Δ mice, and V_κ-J_κ junctions were amplified with either a V_κOx (mouse 45 and 26) or degenerate V_κ primer (mouse 45 and 28) together with a J_κ2 and/or J_κ5 primer. Sequences obtained with the V_κOx-specific primer are aligned with the germline V_κOx1 sequence, and those obtained with the degenerate V_κ primer are each aligned with the most homologous V_κ gene to that gene found in the EMBL database (indicated with their accession number).

Somatic Mutation of κ Genes in Mice Lacking the 3′E_κ

The antibody response to the hapten 2-phenyl-5-oxazolone (ph-Ox) is characterized by the recurrent expression of an LC variable region encoded by a V_κOx1 joined

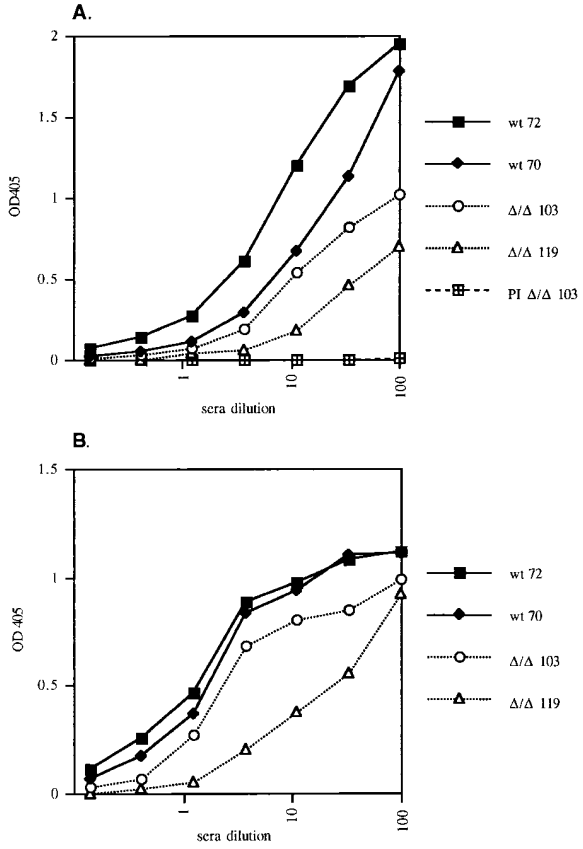

Figure 4. Anti-Oxazolone Antibody Response in 3′E_κ Mutant Mice and Their Wt Littermates

Serum samples were taken before and 7 days after primary immunization (1) and at day 3 after boosting (2), as described in the Experimental Procedures. All sera samples were analyzed for Ox-specific κ-expressing antibodies using oxazolone conjugated to BSA as a capture agent and anti-κ-specific AP-conjugated antibodies for detection as described in the Experimental Procedures. The κ levels are displayed as absorbance units (OD405) versus a. (A) shows the Ox-specific κ response after primary immunization including preimmune serum of 3′E_κΔ/Δ mouse 103. (B) shows the Ox-specific κ response after secondary immunization.

Table 1. Mutation Frequency and Specificity in V_κOx1-J_κ5 Transcripts from Oxazolone-Immunized Mice

3′E_κ Δ/Δ

		Hotspot	Affinity	
V_κOx-clone	# Mut	Ser	His	Tyr
119-a1	5	-	N	F
119-a20	3	-	N	F
119-31	2	-	N	F
119-a33	10	R	N	F
119-a45	4	-	-	F
119-30	6	-	-	-
119-22	6	-	-	-
103-11	9	-	-	-
103-31	10	-	-	-
103-48	0	-	-	-
103-06	0	-	-	-
103-34	1	-	-	-
103-02	2	R	-	-
103-51	5	–	N	F
103-24	4	-	Q	F
103-54	8	-	N	F
103-A1	2	-	-	-
103-A11	11	-	Q	F
103-A13	8	-	Q	F
103-A14	1	-	-	-
103-A15	3	-	-	F

Wt Littermates

		Hotspot	Affinity	
V_κOx-clone	# Mut	Ser	His	Tyr
70-18	11	I	N	F
70-28	13	I	N	F
70-11	1	-	-	-
70-37	5	T	-	-
70-21	10	Y	-	-
70-a16	13	I	N	F
70-a4	12	I	N	F
70-a6	12	Y	-	-
70-A1	8	T	-	-
72-2	4	I	Q	F
72-10	5	-	N	F
72-55	4	-	-	-
72-5	2	-	-	-
72-4	3	-	-	-

Shown is the frequency and nature (e.g., at hotspot or associated with affinity maturation) of mutations in V_κ transcripts derived from oxaolone-immunized mice: 3′E_κ Δ/Δ mice and wt littermates. V_κOx1 transcripts were amplified from B220+/PNA^{high}-expressing splenic B cells and aligned with the published germline V_κOx1 sequence. Transcripts were derived from two 3′E_κΔ/Δ mice (respectively, 119 and 103) and two wt littermates (72 and 70). The total number of mutations are indicated in the first column. Hotspot and affinity maturation mutations in codons 31(Ser), 34(His), and 36(Tyr) are indicated with one letter amino acid code and hyphens indicate no replacement mutation in this codon.

to J_κ5 (Kaartinen et al., 1983; Griffiths et al., 1984; Berek and Milstein, 1988). After secondary immunization, Ox-specific B cells often express V_κOx1 LC genes with an increased number of mutations compared to those seen after the primary response, including frequent mutations (His-34 and Tyr-36) that are known to increase binding affinity (Berek and Milstein, 1987; Alzari et al., 1990; Rada et al., 1991). In addition, several mutational hotspots have been noted (including Ser-26, Ser-28, Ser-31, Met-33, and Ser-77). To assess the role of 3′E_κ in hypermutation of endogenous V_κ-J_κ rearrangements, we analyzed the V_κOx1 response from 3′E_κΔ/Δ and wt littermates following primary and secondary immunization with ph-Ox-chicken-gamma-globulin (CGG). Relative serum anti-Ox-specific κ antibody levels were estimated by ELISA at day 0 and day 7 of the primary immunization and 3 days after boosting. In addition, 3 days after boosting, RNA was prepared from CD45R(B220)$^+$$PNA^{high}$ splenic B cells, V_κOx-J_κ5-specific transcripts were amplified by RT–PCR, and the sequence of at least 20 V_κOx-J_κ5-cDNA clones per mouse was determined.

We analyzed two separate 3′E_κΔ/Δ mice (103 and 119). Both responded to ph-Ox immunization with antigen-specific κ antibodies following primary and secondary immunization; however, the specific antibody levels in the 3′E_κΔ/Δ mice were lower than those of wt mice (Figures 4A and 4B). Although both wt and mutant CD45R(B220)$^+$$PNA^{high}$ B cells expressed a number of

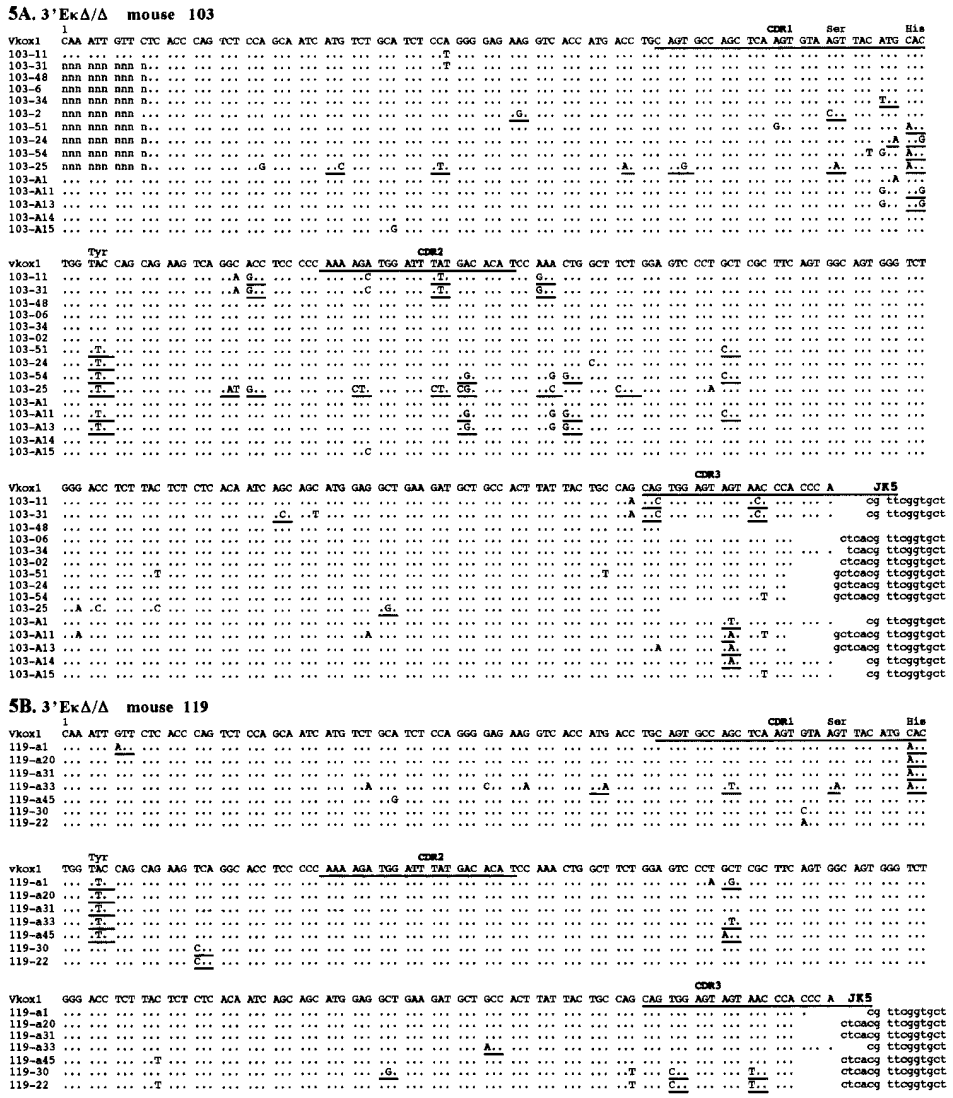

Figure 5. Nucleotide Sequences of Expressed V_κOx1-J_κ5 Transcripts Generated Following Oxazolone

(A and B) Sequences from 3′$E_\kappa\Delta/\Delta$ mice (respectively, 103 and 119). All sequences are aligned with the germline sequence of V_κOx1 (Kaartinen et al., 1983). Since we obtained a large number of identical V_κOx1-J_κ5 transcripts from mouse 119, we sequenced more than 20 clones (38 total) in order to find several different transcripts. Location of Ser−31, His-34, and Tyr-36 are indicated above the germline V_κOx1 sequence. Nucleotide identity is indicated by a period and differences are shown explicitly. CDR regions and replacement mutations are underlined.

different V_κOx family members, we focused our analyses on V_κOx1-J_κ5 transcripts since these have been extensively characterized. V_κOx1-J_κ5 transcripts derived from the 3′$E_\kappa\Delta/\Delta$ cells of mouse 119, which had a lower Ox-specific κ response, also had a low diversity of V_κOx1-J_κ5 transcripts (see Figure 5B legend), while V_κOx1-J_κ5 transcripts derived from the 3′$E_\kappa\Delta/\Delta$ cells of mouse 103, which had a higher response, were more diverse (Table 1 and Figure 5A). Significantly, transcripts from both mutant mice showed evidence of somatic hypermutation, with the total number of mutations per V_κOx1 region showing a similarly broad range to that of transcripts from wt mice (Table 1). Most of the V_κOx1 transcripts in the 3′$E_\kappa\Delta/\Delta$, like those of wt mice, had replacement mutations in His-34 and Tyr-36 (Figures 5A and 5B), suggesting an oxazolone-specific response and normal affinity maturation of the mutant B cells. However, the frequency of the non-antigen-selected Ser-31 mutation appeared lower in V_κOx1 transcripts isolated from 3′$E_\kappa\Delta/\Delta$ mice as compared to those of wt mice (Table 1 and Figure 5), possibly suggesting that antigen-driven B cell activation and/or somatic mutation is less efficient in the mutant cells. In any case, these results clearly demonstrate that the 3′E_κ is not required for significant somatic mutation levels of endogenous κ genes.

Discussion

Previous studies have implicated the 3′E_κ as positively regulating rearrangement, transcription, and somatic mutation of the κ locus in B lineage cells (Meyer et al., 1990; Staudt and Lenardo, 1991; Blasquez et al., 1992;

Meyer et al., 1996). Other studies have concluded that this element also suppresses rearrangement of the κ locus in pro-B cells and in T lineage cells, thereby enforcing stage-specific and lineage-specific rearrangement (Hiramatsu et al., 1995). However, most of these conclusions were reached by studying transgenic κ miniloci. Recently, we have employed mice harboring germline deletions of the 3′$E_κ$ to show that this element plays a significant role in positively regulating the rearrangement and expression of the endogenous κ locus (Gorman et al., 1996). We have now gone on to test directly the proposed functions of the 3′$E_κ$ with respect to lineage- and stage-specific suppression of rearrangement and with respect to regulation of somatic mutation. In contrast to the conclusions of transgenic studies, we find that the 3′$E_κ$ is not required to maintain tissue- and lineage-specific control of the endogenous κ locus. We further show that the 3′$E_κ$ is not required for significant somatic mutation of endogenous κ variable region genes in the context of a specific immune response.

The 3′$E_κ$ Is Not Required to Maintain Lineage- and Stage-Specific Rearrangement of the Endogenous κ Locus

Recent studies of transgenic mice that harbored 2–40 copies of a κ locus rearrangement substrate led to the conclusion that the 3′$E_κ$ acts as a suppressor of $V_κ$-$J_κ$ joining in the context of maintaining B/T-specific and B cell differentiation stage–specific κ LC rearrangement (Hiramatsu et al., 1995; Hayashi et al., 1997). However, we find no evidence for any deregulation of the κ gene rearrangement process in either of these contexts in mice that lack the endogenous 3′$E_κ$ region. In combination with the finding that deletion of the 3′$E_κ$ resulted in a lower level of κ gene rearrangement in λ-expressing B cells (Gorman et al., 1996), our current study demonstrates that this enhancer functions primarily as a positive regulator of rearrangement and has no suppressive function that is unmasked by its deletion.

The discrepancy between our results and those of the prior transgenic studies could be explained by a number of potential difficulties known to be inherent to the transgenic construct approach, including potential absence of additional endogenous regulatory elements (for example, suppressive elements) in the transgenes. In addition, $V_κ$ regions, $J_κ$ regions, promoter, and enhancer elements generally are not organized within transgenic arrays in the same fashion as in the endogenous locus. In particular, promoters may lie closer to enhancers than in the endogenous locus, and the linear organization of elements may be different in transgenic loci due to tandem transgene integration. Such alterations in normal organization could promote functions for elements that may not reflect normal endogenous functions. For example, deregulated transgene expression might be promoted by placing a $V_κ$ gene promoter close to the i$E_κ$ in a tandem array, and such deregulated expression may be disrupted by including another specific regulatory sequence (e.g., 3′$E_κ$) in between. In this regard, different transgenes have given different results. For example, a human transgenic κ rearrangement construct which lacked the 3′$E_κ$ showed appropriate lineage and stage specificity with respect to rearrangement, although this result could have been due to species differences (Cavelier et al., 1997). In any case, our studies clearly show that the 3′$E_κ$ is not required for suppression of endogenous κ rearrangements in murine T cells or in pro-B cells.

The 3′$E_κ$ Is Not Required for Somatic Mutation of Endogenous κ Genes

Analyses of transgenic $V_κ$ constructs have demonstrated that somatic mutation within the constructs was markedly diminished for those that lacked 3′$E_κ$ or the i$E_κ$ (Betz et al., 1994). This enhancer dependence of somatic mutation may be linked to enhancement of transcription, as κ transgene expression levels have been correlated with mutation recruitment (Peters and Storb, 1996; Goyenechea et al., 1997). In agreement with the predicitions of transgene studies, the resting B cells of 3′$E_κ$Δ/Δ mice did have reduced levels of surface κ LC expression (Gorman et al., 1996). However, the 3′$E_κ$Δ/Δ mice, when immunized, generated mutated $V_κ$Ox1 antibodies with mutational patterns characteristic of affinity maturation, indicating no absolute defect in either antigen-specific activation of 3′$E_κ$Δ/Δ B cells or in recruitment of the somatic hypermutation machinery to rearranged κ genes lacking the 3′$E_κ$. This apparent difference in the requirement for 3′$E_κ$ with respect to somatic mutation of the endogenous κ locus versus the transgenic κ loci might reflect transgene-related differences (as outlined above); for example, there may be uncharacterized endogenous elements, redundant in activity to the 3′$E_κ$ with respect to somatic mutation, that were missing from the transgenes. Moreover, the endogenously derived sequences that we characterized, unlike passenger transgene sequences, would have been molded by substantial selection during the immune response. In this context, we do note that mutation frequency of the strong hotspot mutation (Ser-31) in 3′$E_κ$Δ/Δ B cells potentially was decreased in 3′$E_κ$Δ loci, suggesting that the total κ mutational response may be less efficient in the absence of the 3′$E_κ$.

Experimental Procedures

Mice and Immunizations

The mutant mice used in this study harbor a homozygous deletion of the 3′ Ig κ LC enhancer (3′$E_κ$), by replacing this element with either a *loxP* sequence (3′$E_κ$Δ) or a neomycin resistance (*neo*′) gene (3′$E_κ$N) and have been described in a former study (Gorman et al., 1996). Mutant mice and wt littermates were immunized with an intraperitoneal injection of 80 μg of alum-precipitated phOx-CGG (kind gift from G. Kelsoe) and boosted 3 or 7 weeks later with an injection of 80 μg of soluble phOx-CGG. Spleens were removed 3 days after boosting and serum samples were taken before and 7 days after immunization and 3 days after the boost was given.

Serological Assays

Total κ and λ chains were detected by ELISA as described previously (Burstein et al., 1991). Oxazolone-specific κ and λ antibodies were detected by an ELISA using Ox-BSA-coated plates (kind gift from G. Kelsoe) and either anti-κ- or anti-λ-specific AP-conjugated antibodies for detection (Sharpe et al., 1990). All sera samples were analyzed in duplicate wells in 1:3 serial dilutions ranging from 1:100 to 1:72,900.

Immunofluorescence and Cell Sorting

Single-cell suspensions from thymus and spleen were surface stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, biotin-, or Cychrome-C (CyC)-conjugated antibodies and were analyzed by FACScaliber or sorted with the FACStar (Becton Dickinson). The following goat anti-mouse antibodies were used in this study: PE-conjugated anti-λ chain (Southern Biotechnology Associates) and FITC-conjugated anti-κ chain, catalog number 1055–02 (Southern Biotechnology Associates). The following Pharmingen rat anti-mouse antibodies were used: PE- or FITC-conjugated anti-κ chain (R8–140), FITC-conjugated anti-IgM (R6–60.2), CyC-conjugated anti-CD45R/B220 (RA3–6B2), PE-conjugated anti-CD43 (Ly-48), and FITC-conjugated anti-CD3. To isolate activated germinal center B cells after oxazolone immunization, splenic cell suspensions were depleted of erythrocytes and stained with the anti-$B220^{CyC}$ (Pharmingen) and PNA^{FITC} (Pharmingen) and sorted for anti-$B220^+$, PNA^{high}-expressing B cells with the FACS (FACStar, Becton Dickinson).

Splenic B and T cells were sorted from 3′$E_\kappa\Delta/\Delta$ and wt littermates after staining with anti-$B220^{CyC}$ and anti-$CD3^{FITC}$ to obtain purified T and B cell suspension for the analysis of κ rearrangement in B cells versus T cells. To obtain early B cell fractions from the bone marrow, we stained these cells from three 3′$E_\kappa\Delta/\Delta$ mice with anti-$B220^{CyC}$, anti-$CD43^{PE}$, and anti-IgM^{FITC} (Pharmingen) and sorted $B220^+$, $CD43^+$, IgM^- B cells and $B220^+$, $CD43^-$, IgM^- B cells. Thymocytes were purified by staining the thymic cell suspensions with anti-$B220^{FITC}$, anti-IgM^{FITC}, and anti-IgG FITC and depleting the B cells with sheep-anti FITC conjugated bioMags ($BioMag^R$, PerSeptive Biosystems).

Detection of V_κOx-J_κ5 and Total V_κ Gene Rearrangements

Splenic B cells that were $B220^+$, PNA^{high} obtained after immunization were lysed in TRIzol reagent to obtain total RNA samples and used for cDNA synthesis with either a C_κ-specific (Schlissel and Baltimore, 1989) or hexamer primer. V_κOx-J_κ5 regions were amplified by PCR in 30 cycles using specific primers for V_κOx and J_κ5 (Rada et al., 1991). PCR amplifications were done using 1 U of Taq polymerase in 1× PCR buffer (Perkin-Elmer Cetus) and 0.25 mM of each deoxynucleotide triphosphate (dNTP). Each cycle consisted of denaturation at 94°C for 40 s, annealing at 58°C for 40 s, and elongation at 72°C for 1 min and were performed in a 9600 PE thermal cycler (Perkin-Elmer).

Ig κ gene rearrangements in T and B cells were detected by amplifying genomic DNA samples with either a V_κOx-specific or a degenerate V_κ primer (Schlissel and Baltimore, 1989) in combination with a J_κ2 or J_κ5 primer. GADPH control products were amplified using the following primers: GADPHs 5′-AGAAGACTGTGGATGGC CCC- and GADPHas 5′-AGGTCCACCACCCTGTTGC-. Genomic DNA was isolated by lysing the B and T cells in a proteinase-K-containing buffer as described previously (Laird et al., 1991), and PCR reactions were done as described above but with the following cycle conditions: 40′′ at 94°C, 40′′ at 59 °C, and 1′ at 72 °C, preceded by a 2′ denaturation step at 96°C. The cycles were repeated either 30 or 35 times in the 9600 PE thermal cycler. Aliquots of PCR products were separated on an agarose gel and analyzed by Southern blot hybridization (Yancopoulos et al., 1986) using a J_κ1–5 probe (HindIII fragment) (Muller et al., 1990).

V_κ Gene Cloning and Sequence Analyses

Amplified V_κOx-J_κ5 and V_κd-J_κ1–5 or V_κOx-J_κ1–5 regions were subsequently cloned in pT7blue vector (Novagen) and sequenced using either the T7, V_κOx, or J_κ5 primer and sequenase (USB) according to the USB protocol. All obtained V_κOx-J_κ5 sequences were aligned with the known V_κOx1 germline sequence (Kaartinen et al., 1983) and screened for consensus motifs specific for the reported individual V_κOx gene family members (Milstein et al., 1992). In addition, we compared all obtained V_κOx-J_κ5 and V_κd-J_κ1–5 PCR products to the sequences in the EMBL database to search for the most homologous germline (or rearranged) counterpart.

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